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(57) Abstract

A diagnostic test is provided for spongiform encephalopathy and other demyelinating conditions in mammals which comprises assaying antibodies present in the mammal which bind to an antigenic peptide which exhibits molecular mimicry of a mammalian myelin peptide, e.g. one having the sequence FSWGAEGQK. This test is useful for detecting BSE in cattle by assaying sera collected from the cattle for antibodies to a species of Acinetobacter, Agrobacterium or Ruminococcus, or a peptide having a sequence present in said species which mimics a peptide of bovine myelin and identifying animals having a level of antibodies at least about two standard deviations above that of healthy control animals.

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DIAGNOSIS OF SPONGIFORM DISEASE

This invention relates to the detection of spongiform encephalopathy and other demyelinating conditions in mammals and is particularly, but not exclusively, concerned with the diagnosis of bovine spongiform encephalopathy (BSE).

BSE is a recent neurological disorder of cattle, which was first reported in the U.K. after 1982, following a change in the preparation of "bone and meal" feeds. BSE has attracted some public concern, lest it be transmitted to humans following meat consumption. It has been suggested that BSE is caused by "prions", a type of infectious protein.

The present invention is based on an alternative model of the genesis of various forms of spongiform encephalopathy and other demyelinating conditions in mammals. According to the proposed model, BSE and related diseases are conceived as autoimmune diseases arising as a result of molecular mimicry between certain infective agents and the myelin of the infected mammal. This new model of BSE, in particular, is based on the following experimental observations.

A characteristic histopathological feature of BSE is a "spongiform" appearance, which also occurs in chronic but not acute "experimental allergic encephalomyelitis" (EAE), at least in rabbits and guinea pigs. A short sequence of bovine myelin (FSWGAEGQK), which withstands denaturation following heating to 100°C for one hour, was reported over twenty-five years ago to produce hind quarters paralysis, tremors and death, following inoculation into guinea pigs, which to some extent resembles the features observed in cattle suffering from BSE. In accordance with the present invention, this sequence has been used as a computer probe to search for proteins showing molecular mimicry. This sequence, in denatured form, may be described as encephalitogenic.

Analysis of proteins in databases (Genbank and SwissProt) revealed that 3 microbes showed molecular mimicry of the bovine myelin sequence , the best one being found in 4-carboxy-muconolactone-decarboxylase of Acinetobacter calcoaceticus, a common microbe present in soil and water supplies. These sequence similarities are shown in the following Table.

Comparison of amino acids of bovine myelin to microorganisms from Genbank and SwissProt which have similar sequences in other proteins.

Source	Amino acids	Positions	Locations
Bovine myelin	LSRFSWGAE	110 - 118	
Acinetobacter calcoaceticus	ISRFAWGEV	41 - 49	4-carboxy-muconolactone decarboxylase
Agrobacter tumefaciens	YTRFTWGAP	693 - 701	Beta-glucosidase
Ruminococcus albus	YTQFEISAE	274 - 282	Beta-glucosidase

Alphabetic letters refer to biochemical symbols for amino acids.

In conformity with the new model, it has now been found that sera of BSE affected cattle contain significantly high levels of antibodies to Acinetobacter species.

The present invention therefore provides a diagnostic test for spongiform encephalopathy and other demyelinating conditions in mammals which comprises assaying antibodies present in the mammal which bind to an antigenic peptide which exhibits molecular mimicry of a mammalian myelin peptide, especially one having the sequence FSWGAEGQK. The term "molecular mimicry" refers to a degree of similarity (sequence homology) as between the antigenic peptide and a myelin peptide which results in the formation of antibodies which cross-react with myelin and demyelinate nervous tissue. The presence of such antibodies at elevated levels compared to

those found in unaffected animals is therefore a marker for BSE which may be used to detect BSE at an early stage at which curative or other appropriate action may be taken.

The assay may be carried out using the whole Acinetobacter or other organism as the test antigen. Any strain of Acinetobacter having the antigenic peptide identified above may be used. Alternatively the isolated peptide or a synthetic form of the peptide may be used as antigen. Any suitable type of assay procedure may be used, the ELISA method being especially convenient.

Antibody levels indicative of BSE are those which are significantly higher than the control levels. Usually, levels elevated to about 2 standard deviations above the controls may be taken as a positive indication but margins around this figure may be possible or desirable for purposes of caution.

Procedures for carrying out an assay in accordance with this invention are described in the following illustrative Example, based on comparison of sera from animals known to have had BSE with sera from healthy animals.

MATERIALS AND METHODS

Bovine sera

Sera from 29 animals, which were found at post-mortem to satisfy the criteria of BSE and 18 animals which did not, were supplied by the Central Veterinary Laboratory (CVL) (New Haw, Addlestone, Surrey), an executive agency of the U.K. Ministry of Agriculture, Fisheries and Food (MAFF). The 18 animals which did not have BSE had been referred to CVL because of abnormal behaviour but post-mortem examinations carried out by MAFF had excluded BSE.

Furthermore, 30 sera from animals aged less than 30 months (A<30M) (8 Friesians, 21 Hereford-Friesian and 1 Charolais-Friesian crossbreeds) and 28 sera from animals aged more than 30 months (A>30M) (all dairy Friesians), were used as further controls. These were collected

from a farm, kept under "organic farming" conditions where no case of BSE had been reported. Serum samples were obtained during routine herd testing.

Preparation of bacteria

Acinetobacter calcoaceticus was obtained from the National Collection of Industrial and Marine Bacteria Ltd. NCIMB 10694 (Aberdeen). Cultures were grown in 2 l flasks on an orbital shaker for 2 days at 30°C, in 200 ml nutrient broth (Oxoid; 25 g/l). Flasks were inoculated with 10 ml of the corresponding starter culture left shaking at 37°C for 6 hours. Batch culture cells were harvested by centrifugation at 6000 r.p.m. for 20 minutes at 4°C (MSE 18,6 x 250 ml rotor). The pellets of cells were then washed three times with 0.15 M phosphate-buffered saline (PBS; pH 7.4) before being finally resuspended in 20 ml of PBS. A stock solution of the suspension was prepared by diluting in 0.05 M carbonate buffer (pH 9.6) to give an optical density (OD) reading of 0.25 on the spectrophotometer (Corning Model 258).

Enzyme-linked immunosorbent assay

ELISA assays were carried out in the conventional manner. Briefly ELISA plates were coated with bacteria overnight at 4°C and the non-specific sites blocked with PBS containing 0.1% Tween, 0.2% ovalbumin (Sigma, Grade III), plates washed and a 1/200 dilution of test or control serum added. The plates were incubated at 37°C for 1 hour, washed and rabbit anti-cow immunoglobulin (IgG + IgA + IgM) (1:4000) (Dako Ltd.) added. The plates were reincubated for 2 hours, washed and substrate added. The reaction was stopped with a 2 mg/ml solution of sodium fluoride (Sigma). The plates were read at 630 nm on a microtitre plate reader (Dynatech MR 600) and results expressed as OD ± S.E. All studies were carried out under code in that the tester did not know which were test or control sera. The mean OD units of total immunoglobulin antibodies in different groups were compared using Student's t-test.

ELISA METHOD SHEET

1. Dilute antigen in coating buffer, add 200μl to each well. Incubate overnight at 4°C wrapped in foil.
2. Wash out the antigen, using washing/incubation buffer; the wells of the tray should be completely full during the washing stages as the Tween-20 prevents any further protein from being absorbed onto the plastic. Wash 3 times, leaving for approx. 4 minute intervals at room temperature.
3. Incubate the plate at 37°C for 1hr with 0.2% Ovalbumin in washing/incubation buffer.
4. Add 200μl of test serum. Dilutions are made in washing/incubation buffer. Incubate for 2 hours at 37°C wrapped in foil.
5. Repeat washing process as in 2.
6. Add 200μl Horseradish peroxide HRP-conjugated second antibody, also diluted in washing/incubation buffer.
7. Repeat washing process as in 2.
8. Add 200μl substrate (ABTS) to wells; leave to develop colour for approx. 20 minutes in the dark at room temperature. Stop reaction with 100μl of stopping solution and read plate at 630nm.

RESULTS

Antibodies to A. calcoaceticus of total immunoglobulin (IgG + IgA + IgM) were significantly elevated in the BSE sera (mean ± SE: 0.99 ± 0.05) when compared to CVL controls (0.65 ± 0.06) ($t = 4.48$, $p<0.001$), organic farming controls aged more than 30 months (0.57 ± 0.03) ($t = 7.19$, $p<0.001$) and organic farming controls aged less than 30 months (0.53 ± 0.02) ($t = 8.64$, $p<0.001$). These results are shown in the attached Figure.

Legend to figure:

Antibody titres (bar = mean) for 30 controls aged less than 30 months (A<30m), 28 controls aged more than 30 months (A>30m), 18 controls from the Central Veterinary Laboratory (CVL) compared to 29 BSE sera, when tested against Acinetobacter calcoaceticus (Figure la) and E.coli (Figure lb). (Dashed line represents 95% confidence limits for mean of controls: A<30m + A>30m - one tailed test) (OD = optical density).

There was no significant difference between the CVL controls and the organic farming controls aged more than 30 months, but there was a small, statistically significant difference with the sera from animals aged less than 30 months ($t = 2.41$, $p < 0.05$). A re-examination of the CSL control serum with the highest anti-Acinetobacter level of 1.16 OD, showed that it came from a clinically normal control animal, diagnosed as negative to BSE on the statutory diagnostic criteria, and it was also negative when tested for scrapie associated fibrils. This case did however have white matter vacuolation of the substantia nigra and internal capsule, although this had been seen before and not considered significant.

One clear result from these studies, is that in at least in one "transmissible spongiform encephalopathy" (TSE), namely BSE, a specific immune response can be demonstrated against a microbe that is found readily in the environment of cattle and which also happens to possess a molecular sequence resembling bovine myelin.

Other forms of spongiform encephalopathy including Creuzfeld Jacob disease (CJD) and Multiple Sclerosis (MS) are open to explanation on the same model as indicated for BSE. CJD sera and MS sera are currently under test to confirm the presence of cross-reacting antibodies.

CLAIMS

1. A diagnostic test for spongiform encephalopathy and other demyelinating conditions in mammals which comprises assaying antibodies present in the mammal which bind to an antigenic peptide which exhibits molecular mimicry of a mammalian myelin peptide.

2. A test according to Claim 1, in which the mammalian myelin peptide has the sequence FSWGAEGQK.

3. A test according to Claim 1 or 2, for BSE in cattle.

4. A test according to Claim 3, using as the test antigen whole bacteria of an Acinetobacter, Agrobacterium, or Ruminococcus species.

5. A test according to Claim 4, using bacteria of the species Acinetobacter calcoaceticus, Agrobacterium tumefaciens, or Ruminococcus albus.

6. A test according to Claim 3, using as the test antigen a peptide derived from bacteria specified in Claim 4 or 5.

7. A test according to Claim 6, using a peptide of sequence ISRFAWGEV, YTRFTWGAP, or YTQFEISAE.

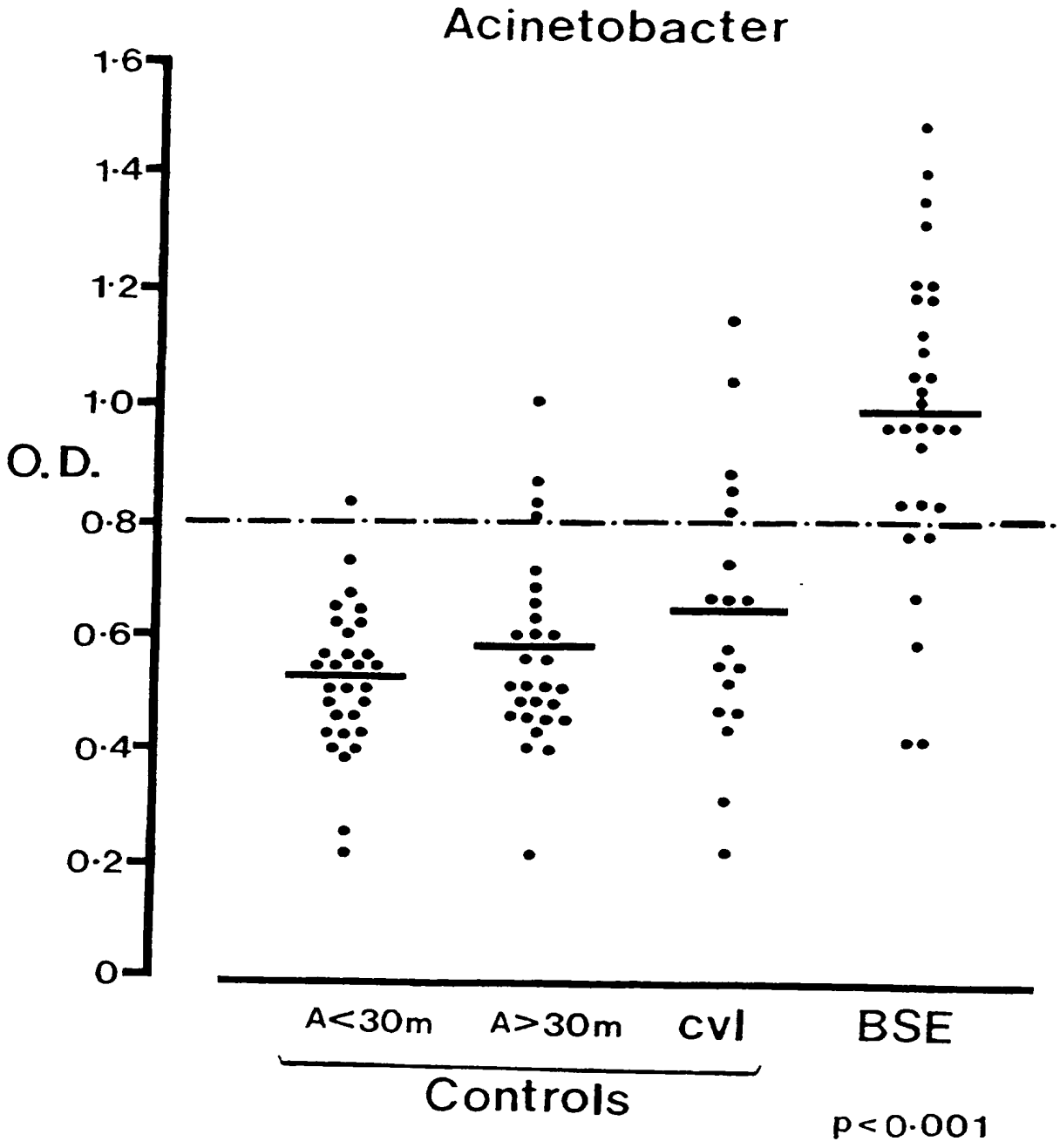
8. A test according to Claim 6 or 7, in which the peptide used is a synthetic peptide.

9. A method of testing for BSE in cattle which comprises assaying sera collected from the cattle for antibodies to a species of Acinetobacter, Agrobacterium or Ruminococcus, or a peptide having a sequence present in said species which mimics a peptide of bovine myelin and identifying animals having a level of antibodies at least about two standard deviations above that of healthy control animals.

10. A method according to claim 9, in which the bovine myelin peptide has the sequence FSWGAEGQK.

11. A diagnostic test kit for BSE in cattle comprising as test antigen a species of Acinetobacter, Agrobacterium or Ruminococcus, or a peptide having a sequence present in said species which mimics a peptide of bovine myelin.

12. A test kit according to claim 11, in which the test antigen is a peptide which mimics the sequence FSWGAEGQK.



INTERNATIONAL SEARCH REPORT

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A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	CHEMICAL ABSTRACTS, vol. 80, no. 11, 18 March 1974 Columbus, Ohio, US; abstract no. 56313, A. WAJGT.: "Assessment by immunofluorescence methods of humoral antimyelin antibody in rats with cyanide encephalopathy." page 68; column 1; XP002052988 see abstract & ANN. IMMUNOL. (POZNAN), vol. 5, no. 1-2, 1973, pages 51-58, --- -/--	1

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex

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INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication,where appropriate of the relevant passages	Relevant to claim No
A	B. H. TOH ET AL.: "The 200- and 150-kDa neurofilament proteins react with IgG autoantibodies from patients with kuru, Creutzfeldt-Jakob disease, and other neurologic diseases." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 82, May 1985, WASHINGTON US, pages 3485-3489, XP002052986 ---	
A	R. L. SIDMAN ET AL.: "Transmissible spongiform encephalopathy in the gray tremor mutant mouse." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA., vol. 82, January 1985, WASHINGTON US, pages 253-257, XP002052987 ---	
A	CHEMICAL ABSTRACTS, vol. 109, no. 21, 21 November 1988 Columbus, Ohio, US; abstract no. 187890, M. P. MCKINLEY ET AL.: "Developmental regulation of prion protein mRNA in brain." page 484; column 2; XP002052989 see abstract & CIBA FOUND. SYMP., vol. 135(Novel Infect. Agents Cent. Nerv. Syst.), 1988, pages 101-116. -----	

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